ANTIBIOTIC SUBSTANCES FROM BASIDIOMYCETES. VIII. PLEUROTUS MULTILUS (FR.) SACC. AND PLEUROTUS PASSECKERIANUS PILAT*

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Several species of the genus *Pleurotus* have been found in this laboratory to form substances inhibitory for *Staphylococcus aureus*. Among these were two species, *Pleurotus mutilus* (Fr.) Sacc. and *P. Passeckerianus* Pilat, obtained from the Centraalbureau voor Schimmelcultures at Baarn. An antibacterial substance formed by these fungi was isolated in crystalline form from culture liquids; it was named pleuromutilin.

P. mutilus grown on corn-steep, thiamine-peptone, or potato-dextrose agars for two days and tested by the streak-method, markedly inhibited *Staphylococcus aureus*, inhibited incompletely *Mycobacterium smegma*, and had no effect on *Escherichia coli*. Agar disks cut from colonies 10 days old formed inhibition zones 20 mm. in diameter with *S. aureus* and a small zone of incomplete inhibition with *M. smegma*. *P. Passeckerianus* produced similar zones of inhibition.

Still Cultures.—*P. mutilus* was grown at 25°C. in 2800 ml. Fernbach flasks containing beech-wood shavings and a corn-steep medium.[1] About four weeks after inoculation the mycelium covered the surface of the liquid and the activity against *S. aureus* was about 512 dilution units per ml. Reflooding the mats with fresh corn steep medium resulted in as high activity in about one week after reflooding. The reflooding was repeated at about 10-day intervals until the mat became so thick that the operation was difficult.

The flasks inoculated with *P. Passeckerianus* reached an activity of 256 dilution units per ml. in about one month.

Shake Cultures and Nitrogen Nutrition.—The fungi were also grown in 200 ml. of solution in 500-ml. Erlenmeyer flasks on a rotary shaker. In the usual corn-steep medium *P. mutilus* liquids developed an activity of 256 dilution units in about 10 days; *P. Passeckerianus* liquids were never more active than 8 dilution units per ml. Since *P. Passeckerianus* was less effective in producing antibacterial material than *P. mutilus*, the latter fungus was used in further work.

In a medium limited to corn-steep and dextrose (omitting the Dox minerals) an activity of from 2000 to 4000 dilution units per ml. was obtained in from 10 to 14 days. By adding single salts of the Dox mixture to the corn-steep-dextrose medium, sodium nitrate was found to be the interfering

substance. Nitrate not only delayed the development of antibacterial activity but also reduced the maximum activity obtained. After more than 15 days' incubation the activity of the medium containing nitrate reached one-half of that of the medium without nitrate. The effect of 9.0 g. of nitrate per liter was greater than that of 3.0 or 6.0 g.

The action of the nitrate was not direct; it appeared to be effective by modifying the metabolism of the fungus. No reduction in activity was found on incubating cultures containing nitrate to which crystalline pleuromutilin had been added. Other nitrogen sources, including ammonium sulfate, asparagine and glycine, had an effect similar to that of nitrate. Glutamic acid substituted for nitrate delayed the development of antibacterial activity but the cultures to which this substance was added eventually reached an activity equal to that of those grown with corn-steep-dextrose medium The addition of nitrate or other nitrogen sources to the corn-steep medium had no marked effect on growth.

The mycelium grown on the shaker contained a small amount of alcohol-soluble antibacterial substance.

No evidence of a change in the capacity of the fungus to produce antibacterial substances was observed in serial transfers. Each flask of a set of flasks was inoculated with several ml. of the mycelial suspension from one flask in the previous set. The final set, representing the tenth transfer from shaker flask to shaker flask during a period of four months, was as active in producing antibacterial material as the first.

Isolation of a Crystalline Antibacterial Substance.—A white, crystalline substance (pleuromutilin) with antibacterial activity was isolated from the culture fluid. The culture fluid was extracted with 0.1 volume of chloroform at pH 5.3, the natural pH of the fluid: the chloroform was evaporated at low temperature. The brown gum remaining was dissolved in ether, the ether solution was extracted once with about 0.05 volume of 1 *N* sodium hydroxide solution to remove acidic substances, washed several times with water, and finally with dilute acid. (Extraction with alkali was not essential for the successful isolation of the active substance.) The ether was evaporated at room temperature and the brown gum allowed to crystallize. The crystalline mass was broken up by stirring with ether, filtered and washed with a small amount of ether. More crystals could be obtained from the filtrate. The brownish crystals were dissolved in a small amount of ethanol in which they were quite soluble: ether and Norit A were added and the solution filtered to remove carbon. The faintly colored solution was evaporated at room temperature to remove the alcohol which interferes with crystallization. The vitreous mass crystallized to form large, clear crystals, if allowed to stand for several days, or if seeded with a few crystals. Usually the mass was dissolved in a minimum of boiling ethanol and on the addition of ether, crystallization occurred.

The white crystals were removed, washed with ether and air-dried. The crystals were so slowly soluble that they could be washed with ether with little loss. About 50 mg. of crystalline pleuromutilin could be obtained from a liter of culture fluid.

White crystals were isolated from the culture fluid of *P. Passeckerianus* by the procedure followed for isolating pleuromutilin. The melting points of these crystals, of pleuromutilin, and a mixture of the two were the same. From this evidence and the relative antibacterial activities, we consider the substance from *P. Passeckerianus* to be pleuromutilin.

Chemical Properties of Pleuromutilin.—The crystals were soluble in alcohol, acetone, chloroform and ether; slightly soluble in water; insoluble in hexane. The melting point in open capillary tubes was 166–169°C. (cor.) without decomposition. The same sample remelted three successive times at 166°. The $[\alpha]_D^{20} \leq 0.05°$ (0.2% in 50% ethanol). The absorption spectrum showed a weak band at 290 mμ (ϵ = 21.7) and end absorption below 230 mμ.

Pleuromutilin was a neutral compound free from halogen, nitrogen, sulfur, ash and without acetyl and methoxyl groups.[2] The molecular weight computed from the depression in freezing point of camphor was 310. Analyses of two lots of crystals were as shown:

	C	H	MOL. WT.
Sample 1	69.78	8.95	..
Sample 2	69.75	9.03	310
Computed for $C_{22}H_{34}O_5$	69.8	8.98	378

Crude pleuromutilin sublimed in the highest vacuum of the oil pump at about 140°C. The condensate did not crystallize and was not pure.

The first crystals were obtained from the contents of the fifth funnel of an eight-funnel counter-current distribution of crude gum between 50% (by volume) aqueous-methanol and a 3:2 mixture by volume of ethyl ether and hexane.

Quantitative acetylation with acetic anhydride in pyridine indicated between one and two acetylatable groups per molecular weight of 378. When bromine in carbon tetrachloride was added to pleuromutilin, hydrobromic acid was released. The permanganate test indicated unsaturation.

Antibacterial activity of pleuromutilin was not changed by boiling for 10 minutes in 0.2 *N* hydrochloric acid, nor in 0.5 *M* sodium bicarbonate (pH 8.8); the antibacterial activity was reduced to 6% of the original activity by boiling in 0.1 *N* sodium hydroxide. The chemical treatment and lack of decomposition on melting indicates that pleuromutilin is stable as compared to most antibiotic substances.

Antibacterial Activity of Pleuromutilin.—The antibacterial activities were measured by the methods used in this laboratory.[3] The results are

expressed as the minimum concentration in micrograms per milliliter that inhibit growth of the test bacteria for 24 hrs.

ANTIBACTERIAL ACTIVITY

Bacillus mycoides	125
Bacillus subtilis	8
Escherichia coli	500
Klebsiella pneumoniae	1
Mycobacterium smegma	32
Pseudomonas aeruginosa	>1000
Staphylococcus aureus	0.25

The activity was mainly against the Gram-positive bacteria.

Pleuromutilin at 0.5 mg./ml. was not antiluminescent when tested against a strain of luminescent bacteria.

The concentration of cells of *S. aureus* in the range from 1000 to 100,000 per ml. did not affect the end-point of the antibacterial tests.

Pleuromutilin was bacteriostatic for *S. aureus* at concentrations between 0.25 and 2 μg./ml. in beef extract medium and bactericidal at concentrations of 4 μg./ml. For concentrations of 4, 8, 16, 32 and 64 μg./ml., all of the bacteria were killed only after 3 days' exposure to pleuromutilin at 36°, although there was substantial reduction in number after 12 hrs.

Pleuromutilin was tested by the paper-disk method[4] for activity against 46 strains of bacteriophages and showed partial activity against only Streptococcus phage 6. Antibacterial activity was detected during the antiphage tests. Pleuromutilin was active against all of the strains of Staphylococcus, Streptococcus, Enterococcus, *B. subtilis*, 80% of the *E. coli* strains, one strain of *Eberthella typhii*, and 60% of the strains of *Vibrio cholera*, and was inactive against one strain of *Bacillus cereus*.

Antifungal Activity.—The antifungal activity of pleuromutilin was measured by the serial dilution method[5] in a peptone medium at pH 6. Pleuromutilin was inactive at 0.5 mg./ml. against *Aspergillus niger*, *Chaetomium globosum* (USDA 1042.4), *Gliomastix convoluta* (PQMD4c), *Memnoniella echinata* (PQMD1c), *Myrothecium verrucaria* (USDA 1334.2), *Penicillium notatum*, *Phycomyces Blakesleeanus*, *Saccharomyces cerevisiae*, *Stemphylium consortiale* (PQMD41b) and *Trichophyton mentagrophytes*.

Effect of Blood.—Incubation for 4 hrs. with 5% whole human blood in beef-extract broth did not reduce the activity of pleuromutilin.

Animal Toxicity.—Toxicity for mice was determined by injecting 0.5 ml. of solutions of pleuromutilin into a tail vein of 14–16 g. Carworth Farms CF1 male mice. The LD 50 of a single dose for mice was greater than 60 mg./kg. Pleuromutilin was given subcutaneously to mice twice daily for three days. There was no evidence of toxicity from a total of 4 mg./mouse.

Activity in Vivo.—Since pleuromutilin was not toxic for mice and could

be given in amounts sufficient to inhibit sensitive bacteria *in vitro*, infected mice[6] were treated. *Streptococcus hemolyticus* C-203 was chosen as the infecting organism since it was inhibited by 0.5 μg./ml. in the presence of rabbit blood. The mice were infected by a dose of one-half million cells given intraperitoneally. The treatment with pleuromutilin was started immediately after infecting the mice. The solution of pleuromutilin was given subcutaneously either as a single dose or as three doses per day for as long as the mice survived. When given as a single dose of pleuromutilin at 50 mg./kg., 63% of the mice were alive 7 days after infecting while 43% survived when given 100 mg./kg. All of the penicillin treated and 20% of the untreated controls were alive at 7 days. All of the mice died within 7 days when they were treated three times daily with either 25 mg./kg. or 50 mg./kg. doses of pleuromutilin.

Pleuromutilin at a daily dose of 400 mg./kg. did not retard the growth of sarcoma 180 in mice.[7] Pleuromutilin was inactive against *Plasmodium gallinaceum* at 40 mg./kg. when administered orally twice daily for four days.[8]

Repeated contact with solutions or solid pleuromutilin caused mild urticaria in susceptible individuals.

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[1] The usual corn-steep medium contained, per liter, 1.5 g. KH_2PO_4, 0.5 g. KCl, 0.5 g. $MgSO_4$ $7H_2O$, 3 g. $NaNO_3$, 40 g. dextrose, and 5 g. Staley special nutrient 114 (corn steep).

[2] The analyses were made by Mr. J. F. Alicino and by The Huffman Analytical Laboratories.

[3] Kavanagh, F., *Bull. Torrey Bot. Club*, **74**, 303–320 (1947).

[4] Asheshov, I. N., Strelitz, F., and Hall, E., *Brit. Jour. Exptl. Path.*, **30**, 175–185 (1949).

[5] Anchel, M., Hervey, A., Kavanagh, F., Polatnick, J., and Robbins, W. J., these PROCEEDINGS, **34**, 498–502 (1948).

[6] Through the courtesy of Dr. W. C. Robbins, of Cornell Medical School, infected mice were treated with pleuromutilin.

[7] Tests made through the courtesy of Dr. Chester Stock of Sloan-Kettering Institute for Cancer Research.

[8] Tests made through the courtesy of Dr. Joseph Greenberg of the National Institutes of Health.